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ENDOGENOUS PROTEASE ACTIVITY IN BY-PRODUCTS OF PINK SALMON (*ONCORHYNCHUS GORBUSCHA*)

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ABSTRACT

*Hydrolysate production is a low-cost method of preservation that could be employed to decrease the amount of fish by-products discarded by Alaska's salmon industry. However, endogenous enzymes within salmon vary with spawning maturity, and must be controlled in the raw material to ensure a consistent hydrolysate. Differences in proteolytic activities were found among tissue groups (fillets, heads, livers and viscera) in male and female adult pink salmon (*Oncorhynchus gorbuscha*) harvested at three different levels of spawning maturity. Changes in protease levels may have implications for processing hydrolysates when pink salmon of different maturity levels are used.*

PRACTICAL APPLICATIONS

Proteases and other enzymes commonly found in raw fish can interfere with production of a consistent hydrolysate. This study demonstrated that endogenous proteases in pink salmon varied among different tissues as the fish moved from ocean to river environments for spawning. Variation in proteolytic activities among fish tissues will have implications for processors who use different maturity levels of pink salmon to produce hydrolysates, since by-products from a roe-stripped carcass will contain different protease levels than by-products taken from ocean-harvested salmon.

INTRODUCTION

Alaska is credited with harvesting over half of the U.S.A.'s total fish, about 9% of which is salmon (Crapo and Bechtel 2003). By-products from

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Alaska's fishing industry represent over one million metric tons of waste, some of which is processed into fish meal. However, due to the seasonal nature of the salmon harvest (June through September) and the remote locations of the catch, unused portions of fish may not be fully utilized. Common methods of waste disposal include grinding and dumping the discarded fish back into the ocean, despite the loss of valuable marine oils and proteins. Production of hydrolysates is a low-cost method of preservation that could be employed to decrease the amount of high-quality fish protein discarded (Shahidi and Kamil 2001). However, to prepare a consistent hydrolysate product, the endogenous proteases and other enzymes in the raw material may need to be controlled (Sikorski and Naczk 1981; Kristinsson and Rasco 2000).

Proteolytic enzymes catalyze peptide bonds and are abundant in fish. Proteases include digestive enzymes (such as pepsin, trypsin, chymotrypsin and elastase) as well as muscle proteases (such as cathepsins and calpains), which play an active role in postmortem degradation of tissue (Kristinsson and Rasco 2000; Mommsen 2004). The ratio of these enzymes varies during spawning migration in salmon as their energy needs change. Energy in migrating salmon must be expended toward gonadal development, which can constitute 20% of body mass (Hendry *et al.* 1999). Energy must also be allocated for migration from the ocean to the spawning grounds, as well as competition for nesting sites and defense of the eggs (Hendry *et al.* 2000). Despite this high demand for energy during their reproductive cycle, the salmon stop feeding when they transition from ocean to river water and rely entirely on their stored energy (Brett 1995).

Since there are many endogenous proteases that change in both quantity and importance during spawning, detection of these enzymes requires multiple assays targeted at different pH optima to detect major changes in proteolytic activity in migrating salmon. Proteases that display activity in acid environments (pH 4.0) include lysosomal catheptic enzymes, which are involved in protein catabolism during spawning migration of salmon (Yamashita and Konagaya 1990). Proteases with higher pH optima include trypsin-like enzymes, which participate in degradation of fish muscle and are most active near pH 7.3 (Kolodziejska and Sikorski 1997), but are unstable in acidic medium (Kolodziejska and Sikorski 1996).

Proteolytic activity can be classified into three different stages associated with spawning: ocean bright (a premium quality fish with silver scales and no watermark), mature (a fish displaying prominent watermarks and some loss of scales) and spawning (a heavily watermarked fish with lost scales, where males display hooked mouths and large humps). The salmon were subdivided into four different tissues (fillets, heads, livers and viscera) for comparison of proteolytic activity. The objective of this study was to evaluate the change in

product quality of pink salmon destined for use in hydrolysates, based on the spawning maturity of the fish at time of harvest.

MATERIALS AND METHODS

Sampling Methods

Pink salmon (*Oncorhynchus gorbuscha*) were harvested near Kodiak, Alaska in August 2005. Fish were sorted visually for differences in appearance relating to quality: Bright – best quality ocean fish obtained from a commercial processor; Mature – very water marked, less firm texture, low-quality fish with loss of scales obtained from a commercial processor; Spawning – river fish, large humps and hooked mouths on males, mature large roe in females, loss of scales, very watermarked, fillets much less firm, pale white color, liver also appeared less dark, with both liver and viscera appearing smaller than Brights and Matures and large amounts of light brown muscle layer below skin were captured from a river. Samples taken from each fish included head, liver, fillets and viscera. Heads were obtained by cutting the salmon directly behind the gills. Attached pieces of liver were removed, although salmon hearts were left inside the head. Liver samples were removed whole whenever possible and the gall bladder was removed when identified. Viscera samples were collected after removing liver, roe and/or milt. One fillet was removed using standard procedures with slight trimming to remove dark muscle from under skin.

Sampling included three female and three male salmon from each maturity class (Bright, Mature and Spawning). All parts of the same fish were labeled with the same number, individually vacuum packaged and frozen (–30C). The experimental design included three replicates, two sex types, three spawning maturity classes, four tissues and three protease assays.

Compositional Analysis

Three samples from each treatment method were analyzed in duplicate for moisture, protein, lipids and ash. Moisture was determined gravimetrically by drying samples at 103C for 24 h and measuring water loss (method 952.08, AOAC 1990). Protein was measured by drying samples (103C, 24 h) and analyzing for nitrogen content on an Elementar Rapid NIII analyzer (Mt. Laurel, NJ) using WINRAPID software to calculate protein values. Concentrations of soluble proteins were determined by the method of Bradford (1976). Lipids were determined by processing dried samples on a Soxtec Model 2043 (Foss Analytical, Hillerød, Denmark) using a methylene dichloride extraction solvent, then evaporating lipid-rich solutions to dryness to remove solvent before weighing. Ash content was determined by placing

samples into a muffle furnace at 550C for 24 h, and then weighing the remaining material (Method 938.08, AOAC 1990).

Protease Assays

Crude proteolytic enzymes were obtained from aqueous extracts of each tissue sample. All samples were kept frozen until processing and then homogenized at 4C with three volumes of cold distilled water using an Ultra-Turrax homogenizer (IKA-Werke, Staufen Germany). The homogenates were centrifuged at 5,000 × g (20 min, 4C), then the supernatants were removed and frozen at −80C until tested. Relative quantities were determined based on their hydrolyzing activities toward substrates at three different pH levels. All assays were conducted at 35C and all proteases recovered were tested without further purification.

Different hydrolyzable substrates were prepared to cover the pH optima of three major protease groups found in fish: pH 3.5 (cathepsin D, white muscle and acid proteases), pH 6.5 (neutral proteases), pH 7.3 (trypsin-like enzymes and muscle proteases). To evaluate protease activity at pH 6.5 and pH 7.3, caseinolytic assays were run (Rick 1974). The acid protease assay (pH 3.5) was based on the method of Rick and Fritsch (1974), with slight modifications to the system (2% hemoglobin in acetate buffer) adapted from Barrett (1970). Absorbances of crude protease solutions were measured using a SpectroMax Plus microplate spectrophotometer (Molecular Devices, Union City, CA) at 280 nm. An internal standard of trypsin was run with each assay so that all activities could be reported in units of trypsin activity. Trypsin, powdered hemoglobin from bovine blood, casein, and Folin and Ciocalteu's phenol reagent were purchased from Sigma (Sigma-Aldrich-Inc., St. Louis, MO).

Statistical Analysis

The effects of all treatments were investigated using one-way analysis of variance (ANOVA) conducted with the Statistica v 7.1 software package (Statsoft, Tulsa, OK). The ANOVA P value was set to 0.05, and differences between treatments were examined using the post hoc test Tukey's honestly significant differences ($P < 0.05$).

RESULTS AND DISCUSSION

Composition

Pink salmon representing three different stages of spawning maturity (ocean bright, mature and river fish just prior to spawning) were analyzed for

moisture, protein, fat and ash composition (Table 1). Large variations among fish were observed, even within each treatment group (Bright, Mature and Spawning), which is a trend previously noted by researchers working with salmon (Hendry *et al.* 2000). The amount of moisture in fillets was similar among ocean bright salmon and mature treatment groups. However, statistically lower moisture levels were observed between mature female fillets and spawning male fillets (Table 1). No differences were found in the moisture content of the liver, regardless of gender or spawning maturity of the salmon. Moisture within salmon viscera showed some variation, but values were generally not statistically significant. However, salmon heads displayed differences between males and females and between spawning maturity groups. Heads from ocean bright and mature groups possessed significantly less moisture than their spawning counterparts.

The protein content within individual salmon fillets was also highly variable and showed few statistically significant differences (Table 1). Ocean-harvested females had significantly more protein in their fillets than the males. However, female salmon fillets decreased in protein with spawning maturity, whereas male salmon fillets remained fairly constant in protein values. Protein content in salmon livers ranged from 15.8 to 17.7%; however, statistically significant differences appeared only in female salmon as they approached spawning maturity. Differences in the protein levels within the viscera did not significantly vary among salmon regardless of age or gender of the fish; however, there were larger standard error values than seen in the other analyzed tissues. Salmon heads also displayed few differences in protein quantity among fish of different spawning maturities.

Lipid levels generally varied among ocean bright, mature and spawning salmon (Table 1). However, in fillets, no significant differences were observed between genders, nor were any identifiable trends associated with the different ages of the fish. Lipid levels were statistically different within the livers, decreasing in quantity as salmon approached spawning maturity. Differences between genders also were apparent, with ocean bright and spawning females having significantly higher liver-lipid levels than their male counterparts. Salmon viscera displayed gender differences in lipid quantities for ocean bright and mature groups. Salmon heads from ocean-harvested fish had lipid levels that were significantly higher than those found in the heads of river salmon just spawning group, regardless of gender. These results are supported by Mommsen (2004), who followed sockeye salmon (*Oncorhynchus nerka*) during their 1150 km spawning migration and noted that the fish lost nearly all their lipid during the migration. This lipid decrease directly corresponds with the increase in moisture that occurred as the fish approached spawning.

Ash levels in fillets and viscera generally did not differ among salmon, regardless of gender or spawning maturity (Table 1) and were comparable to

TABLE 1.
EFFECT OF SPAWNING MATURITY ON THE COMPOSITION OF PINK SALMON FILLETS, LIVER, VISCERA AND HEADS FOR FEMALE (F) AND MALE (M) FISH

a. Moisture (%)

	Fillets	Liver	Viscera	Heads
Ocean Bright				
F	74.6 (±0.6)^{abc}	77.3 (±0.6)^{a}	81.6 (±0.7)^{b}	68.5 (±1.2)^{a}
M	75.2 (±0.6)^{abc}	77.3 (±0.2)^{a}	78.6 (±0.2)^{a}	73.2 (±0.7)^{b}
Mature				
F	73.3 (±1.4)^{a}	77.6 (±0.7)^{a}	78.2 (±0.7)^{a}	72.8 (±1.3)^{b}
M	73.9 (±0.9)^{ab}	78.7 (±1.0)^{a}	80.6 (±0.8)^{ab}	75.8 (±0.6)^{bc}
Spawning				
F	77.0 (±0.6)^{bc}	77.3 (±0.6)^{a}	79.8 (±0.3)^{ab}	78.7 (±0.6)^{cd}
M	78.0 (±0.5)^{c}	79.6 (±0.3)^{a}	80.2 (±0.7)^{ab}	81.8 (±0.5)^{d}

b. Protein (%)

	Fillets	Liver	Viscera	Heads
Ocean Bright				
F	21.2 (±0.4)^{c}	16.1 (±0.4)^{ab}	14.3 (±0.1)^{a}	12.4 (±0.5)^{a}
M	16.4 (±0.7)^{ab}	16.9 (±0.2)^{abc}	14.6 (±0.2)^{a}	12.9 (±0.3)^{ab}
Mature				
F	19.3 (±1.0)^{bc}	17.6 (±0.6)^{bc}	16.9 (±1.3)^{a}	12.7 (±0.4)^{a}
M	20.0 (±1.6)^{bc}	15.8 (±0.3)^{a}	18.2 (±0.9)^{a}	14.4 (±0.4)^{b}
Spawning				
F	14.9 (±0.5)^{a}	17.7 (±0.3)^{c}	15.6 (±0.8)^{a}	11.9 (±0.5)^{a}
M	17.8 (±0.9)^{abc}	16.0 (±0.0)^{a}	12.9 (±3.0)^{a}	11.4 (±0.3)^{a}

c. Lipid (%)

	Fillets	Liver	Viscera	Heads
Ocean Bright				
F	2.6 (±0.3)^{ab}	2.5 (±0.1)^{d}	1.2 (±0.1)^{ab}	11.9 (±0.6)^{d}
M	3.8 (±0.6)^{b}	1.9 (±0.3)^{c}	2.7 (±0.4)^{c}	10.5 (±0.2)^{cd}
Mature				
F	2.2 (±0.4)^{ab}	1.1 (±0.2)^{b}	1.8 (±0.2)^{b}	9.1 (±1.0)^{c}
M	1.6 (±0.2)^{a}	0.9 (±0.1)^{ab}	1.0 (±0.1)^{a}	6.0 (±0.4)^{b}
Spawning				
F	2.0 (±0.6)^{a}	1.4 (±0.1)^{bc}	1.1 (±0.0)^{ab}	4.8 (±0.8)^{ab}
M	3.1 (±0.3)^{ab}	0.4 (±0.1)^{a}	0.9 (±0.1)^{a}	2.5 (±0.2)^{a}

d. Ash (%)

	Fillets	Liver	Viscera	Heads
Ocean Bright				
F	1.5 (±0.1)^{b}	5.0 (±0.1)^{c}	1.4 (±0.1)^{a}	2.0 (±0.2)^{a}
M	1.4 (±0.1)^{b}	5.1 (±0.1)^{c}	1.3 (±0.1)^{a}	2.3 (±0.2)^{a}
Mature				
F	1.4 (±0.1)^{b}	2.8 (±0.7)^{b}	1.5 (±0.1)^{a}	2.6 (±0.1)^{a}
M	1.5 (±0.1)^{b}	2.2 (±0.5)^{ab}	1.4 (±0.1)^{a}	2.4 (±0.1)^{a}
Spawning				
F	1.4 (±0.1)^{b}	1.3 (±0.1)^{a}	1.8 (±0.4)^{a}	2.3 (±0.1)^{a}
M	1.2 (±0.1)^{a}	1.3 (±0.1)^{a}	1.3 (±0.5)^{a}	2.0 (±0.2)^{a}

Values for moisture, protein, lipid and ash are expressed as percentages with standard errors in parentheses. Different letters within a column indicate statistical difference at $P < 0.05$.

previously measured quantities of ash (1.4% in fillets and 1.2% in viscera) for salmon (Hendry *et al.* 2000). Salmon heads showed no variation in ash levels, although salmon livers significantly decreased in ash from ocean bright fish to mature to spawning fish in both genders.

Proteolytic Activity

Proteolytic activities within different salmon tissues are shown in Table 2. Muscle tissue in salmon fillets displayed increasing quantities of proteolytic enzymes (per mg of soluble protein) at all three pH levels as the fish moved from ocean bright to spawning groups (Table 2). For female salmon, this increase in enzyme activity correlated with the muscle proteolysis observed as protein levels decreased in the ocean-dwelling fish from 21 to 15% in spawning group (Table 1). This finding is consistent with Stoknes and Rustad (1995) and Yamashita and Konagaya (1990), who noted that muscle texture degradation in mature salmon was associated with increased proteolytic activity. Proteolytic activity in the fillets of male salmon also increased with spawning maturity, but protein content did not decrease.

Salmon viscera contained the largest quantities of proteolytic enzymes at all three pH levels. Protease values for spawning salmon appeared to be lower per mg of soluble protein than for ocean bright, although these differences were generally not significant (Table 2). Carnivorous fish, such as salmon should have high levels of proteases such as pepsin in their digestive system, resulting in large proteolytic activities in their viscera. However, salmon are anadromous fish and stop feeding during spawning migration, which requires catabolism of fats and protein to meet energy demands (French *et al.* 1983). Consequently, protease levels would be expected to decrease in the viscera, as shown in Table 2. However, large variations in protease were observed in the viscera of individual ocean bright salmon. This was of interest since protease values for ocean bright livers did not exhibit a large standard error. This variation in visceral protease values was due in part to the diversity of visceral components (e.g., stomach, intestines, air bladder), which may differ in quantity from fish to fish. Additionally, silver ocean salmon were likely still feeding, unlike their more mature (spawning) counterparts. Since the initial stomach contents were not evaluated, error may have been introduced when each salmon's last meal was inadvertently included in the analysis.

Proteolytic activity in the liver significantly increased at all three pH levels for females as they approached spawning maturity, although fewer differences were seen among male salmon (Table 2). Biochemical changes are known to occur within specific organs during spawning migration of sockeye salmon. For example, liver cells in spawning females were found to have high rates of activity throughout the migration (French *et al.* 1983). Salmon livers

TABLE 2.
EFFECT OF SPAWNING MATURITY ON PROTEOLYTIC ACTIVITIES FROM FILLETS, VISCERA, LIVER AND HEADS IN FEMALE (F) AND MALE (M) PINK SALMON

a. Fillets

	pH 3.5	pH 6.5	pH 7.3
Ocean Bright			
F	75.4 (±0.1)[a]	58.3 (±0.2)[a]	60.0 (±0.1)[a]
M	82.2 (±1.4)[ab]	59.5 (±3.3)[a]	63.5 (±2.1)[ab]
Mature			
F	91.7 (±4.3)[b]	67.7 (±4.6)[ab]	72.1 (±4.6)[ab]
M	83.5 (±3.3)[ab]	62.6 (±2.0)[a]	63.3 (±1.1)[ab]
Spawning			
F	106.4 (±3.5)[c]	78.2 (±3.6)[bc]	77.1 (±4.6)[cd]
M	117.1 (±2.0)[c]	83.7 (±3.0)[c]	89.1 (±2.5)[d]

b. Viscera

	pH 3.5	pH 6.5	pH 7.3
Ocean Bright			
F	242.6 (±41.2)[ab]	318.1 (±101.4)[a]	362.8 (±100.3)[bc]
M	288.2 (±23.4)[b]	302.0 (±0.9)[a]	368.3 (±25.4)[c]
Mature			
F	325.6 (±22.5)[b]	317.4 (±42.9)[a]	436.3 (±39.1)[c]
M	164.7 (±9.7)[a]	124.0 (±10.6)[a]	142.1 (±9.1)[a]
Spawning			
F	175.7 (±5.9)[a]	125.4 (±0.5)[a]	129.2 (±1.4)[a]
M	230.4 (±17.2)[ab]	126.1 (±8.8)[a]	147.3 (±11.7)[ab]

c. Liver

	pH 3.5	pH 6.5	pH 7.3
Ocean Bright			
F	61.4 (±2.4)[a]	44.3 (±1.0)[a]	58.0 (±4.6)[a]
M	56.9 (±2.0)[a]	50.3 (±4.4)[abc]	59.1 (±1.7)[a]
Mature			
F	67.8 (±3.0)[a]	49.0 (±0.5)[ab]	53.9 (±0.9)[a]
M	63.4 (±3.7)[a]	56.0 (±3.4)[bc]	66.1 (±3.6)[a]
Spawning			
F	124.2 (±11.0)[b]	81.8 (±1.7)[d]	90.9 (±1.4)[b]
M	76.8 (±2.5)[a]	61.7 (±0.3)[c]	66.1 (±0.5)[a]

d. Heads

	pH 3.5	pH 6.5	pH 7.3
Ocean Bright			
F	75.2 (±10.4)[b]	54.9 (±6.8)[b]	56.4 (±6.9)[b]
M	44.6 (±2.2)[a]	34.1 (±1.7)[a]	35.5 (±1.7)[a]
Mature			
F	51.6 (±3.9)[a]	39.8 (±2.5)[ab]	41.1 (±1.6)[ab]
M	50.6 (±2.0)[a]	39.3 (±1.1)[ab]	40.9 (±1.9)[ab]
Spawning			
F	50.6 (±1.3)[a]	50.7 (±6.2)[ab]	49.2 (±4.5)[ab]
M	46.0 (±1.5)[a]	46.7 (±2.0)[ab]	46.1 (±0.7)[ab]

Values are expressed as the mean of proteolytic enzyme per mg soluble protein with standard errors in parentheses. Different letters within a column indicate statistical difference at $P < 0.05$.

are also known to contain cathepsin D, a proteolytic enzyme that contributes to muscle-softening during spawning migration (Hansen *et al.* 1996), so increased levels of protease were not unexpected.

Heads from mature and spawning salmon had no significant differences in proteolytic activity at any of the three pH levels tested (Table 2). However, female and male ocean bright fish were statistically different at each pH, with females containing consistently higher quantities of proteolytic enzymes.

This study demonstrated that endogenous proteases vary within different pink salmon tissues during spawning migration. For example by-products taken from a roe-stripped carcass will contain different quantities of protease than by-products taken from ocean-harvested salmon. These differences in proteolytic activity will have implications for processors who combine different maturity levels of pink salmon when preparing hydrolysates.

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